OPEN ACCESS

Orapuh Journal

ORIGINAL ARTICLE

Journal of Oral & Public Health

Optimization of granulation mechanisms and microbiological characterization of anaerobic granules in hospital wastewater treatment using a UASB reactor enriched with natural additives

Mesongolo, C. L.[1], Musibono, D. E.[1], Tangou, T. T.[1], Mulaji, C. K.[2], Seke, M. V.[3], Asamboa, L. S.[4], & Biey, E. M.[1]

[1]*Laboratory of Ecotoxicology, Chemical Safety, and Environmental Biotechnology, Department of Environmental Science and Management, Faculty of Science and Technology, University of Kinshasa, Kinshasa, Democratic Republic of the Congo* [2]*Department of Chemistry and Industry, Faculty of Science and Technology, University of Kinshasa / Department of Geostrategy and Environment, Center for Research in Human Sciences (CRESH), Kinshasa, Democratic Republic of the Congo* [3]*Department of Physics and Technology, Faculty of Science and Technology, University of Kinshasa, Kinshasa, Democratic Republic of the Congo* [4]*Laboratory of Applied Microbiology of Biological and Natural Resources (LaMARBN), Department of Life Sciences, Faculty of Science and Technology, University of Kinshasa, Kinshasa, Democratic Republic of the Congo*

ARTICLE INFO

Received: *13 January 2026*
Accepted: *13 February 2026*
Published: *14 July 2026*

Keywords:
UASB, anaerobic granulation, cultivable microbial diversity, CFU/mL, hospital wastewater, natural additives

Peer-Review: *Externally peer-reviewed*

Correspondence to:
Dr. Credo Lialia Mesongolo
credo.mesongolo@unikin.ac.cd

To cite:
Mesongolo, C. L., Musibono, D. E., Tangou, T. T., Mulaji, C. K., Seke, M. V., Asamboa, L. S., & Biey, E. M. (2026). Optimization of granulation mechanisms and microbiological characterization of anaerobic granules in hospital wastewater treatment using a UASB reactor enriched with natural additives. *Orapuh Journal, 7*(5), e1449. https://doi.org/10.4314/orapj.v7i5.49

ISSN: *2644-3740*

Published by ***Orapuh, Inc.***, F. Gaye Res., Sukuta-Jamisa, Greater Banjul, The Gambia.

Editor-in-Chief: *Prof. V. E. Adamu (editor@orapuh.org)*

ABSTRACT

Introduction
Anaerobic granulation is a critical process governing biomass retention, hydrodynamic stability, and treatment efficiency in Upflow Anaerobic Sludge Blanket (UASB) reactors treating high-strength wastewaters. However, limited empirical evidence exists regarding the microbiological structure and granulation mechanisms of anaerobic granules developed during the treatment of hospital wastewater, particularly under conditions enhanced by natural additives in low-resource settings.

Purpose
This study aimed to quantitatively and qualitatively characterize the cultivable microbial community associated with anaerobic granules formed in an optimized UASB reactor treating raw hospital wastewater from the University Clinics of Kinshasa, with specific attention to granulation mechanisms promoted by natural additives.

Methods
A pilot-scale UASB reactor was operated under steady-state conditions and supplemented with natural additives (natural clay, crushed eggshells, ground maize, and lime) to enhance microbial activity and granule formation. Anaerobic granules were aseptically collected and microbiologically analyzed using selective and non-selective culture media at a 10^{-1} dilution. Microbial enumeration was performed in triplicate and expressed as colony-forming units per milliliter (CFU/mL). Morphological and structural characteristics of the granules were assessed via optical microscopy.

Results
The total cultivable microbial density reached 1.65 x 10^4 CFU/mL. Fermentative bacteria predominated, representing approximately 76% of the cultivable population. Biofilm-forming bacteria, including staphylococci and *Pseudomonas aeruginosa*, collectively accounted for about 30%, while hydrolytic fungi represented approximately 16.5%. Microscopic observations revealed compact, spherical granules (1–3 mm diameter) with distinct microbial stratification and a well-developed extracellular polymeric substance (EPS) matrix, indicating stable anaerobic granulation.

Conclusions
The findings demonstrate that natural additives can effectively promote microbial growth, EPS production, and structural stability of anaerobic granules during hospital wastewater treatment in UASB reactors. This study provides practical insights into granulation mechanisms under real hospital effluent conditions. Future research integrating molecular microbial analyses and long-term reactor performance monitoring is recommended to better elucidate microbial dynamics and system resilience.

INTRODUCTION

Hospital wastewater represents a major environmental and public health concern due to its complex and heterogeneous composition, which typically includes high organic loads, pathogenic microorganisms, pharmaceutical residues, and potentially toxic chemical substances (Verlicchi et al., 2010). In many developing countries, particularly in sub-Saharan Africa, these effluents are often discharged into natural water bodies with little or no prior treatment, thereby increasing the risk of antimicrobial resistance dissemination, ecosystem degradation, and human exposure to waterborne pathogens.

Among available biological treatment technologies, Upflow Anaerobic Sludge Blanket (UASB) reactors are widely recognized as efficient and cost-effective options for treating high-strength wastewaters, especially under tropical and subtropical conditions (Lettinga et al., 1997). Reactor performance relies primarily on the formation and maintenance of anaerobic granules. These granules are dense, self-immobilized microbial aggregates capable of retaining high biomass concentrations, enhancing process stability, and improving organic matter conversion efficiency.

Anaerobic granulation is a complex and dynamic process governed by multiple interacting factors, including substrate composition, hydrodynamic shear forces, pH, alkalinity, ionic strength, and microbial community structure and interactions (Hulshoff Pol et al., 2004). Previous studies demonstrate that fermentative bacteria play a central role in acidogenesis, while extracellular polymeric substance (EPS)-producing microorganisms facilitate cell aggregation and adhesion. In parallel, biofilm-forming bacteria contribute to granule cohesion, structural integrity, and resistance to hydraulic stress (Flemming & Wingender, 2010; Liu et al., 2004).

In recent years, increasing attention has been directed toward using natural additives—such as mineral supports, biodegradable organic substrates, and pH-regulating materials—as a strategy to enhance anaerobic granulation and UASB reactor performance (Jiang et al., 2016). Mineral-based materials can act as nucleation sites for microbial attachment, calcium-rich additives promote granule densification and buffering capacity, and readily biodegradable substrates stimulate microbial growth and EPS synthesis. Despite these promising findings, quantitative microbiological characterization of anaerobic granules formed during hospital wastewater treatment remains limited, particularly in Central African settings where wastewater composition, climatic conditions, and operational constraints differ markedly from those reported in temperate regions.

To address this knowledge gap, the present study provides a culture-based microbiological characterization of anaerobic granules developed in an optimized UASB reactor treating raw hospital wastewater from the University Clinics of Kinshasa. The study focuses on the enumeration, relative abundance, and functional roles of major cultivable bacterial and fungal groups involved in granulation mechanisms under the influence of natural additives, namely natural clay, crushed eggshells, ground maize, and lime. This research was guided by the hypothesis that the combined application of mineral and organic natural additives enhances anaerobic granulation by selectively promoting fermentative, EPS-producing, and biofilm-forming microorganisms, thereby improving granule stability and functional microbial diversity.

Study Objectives

General Objective

To investigate the effect of natural additives on microbial diversity and granulation mechanisms in a UASB reactor treating hospital wastewater.

Specific Objectives

i. To identify and enumerate the major cultivable microbial groups present in anaerobic granules;

ii. To quantify total microbial density expressed as colony-forming units per milliliter (CFU/mL);

iii. To assess the relative dominance of key functional microbial groups involved in granulation;

iv. To elucidate the functional roles of bacterial and fungal populations in anaerobic granulation processes.

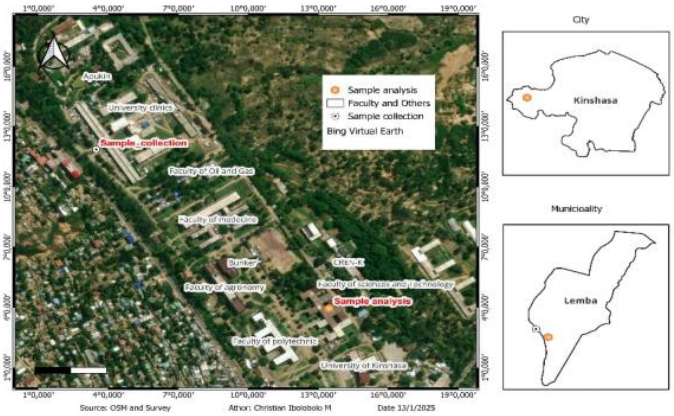
https://doi.org/10.4314/orapj.v7i5.49

METHODS

Study Site and UASB Reactor Configuration

The study was conducted at the University Clinics of Kinshasa (CUK), a major tertiary healthcare facility located in Kinshasa, Democratic Republic of Congo **(Map 1)**. The hospital generates complex wastewater streams originating from clinical wards, diagnostic laboratories, and sanitary facilities. A pilot-scale UASB reactor was installed on-site and continuously fed with raw hospital wastewater without prior physicochemical treatment.

Map 1:
View of the University Clinics of Kinshasa and the Faculty of Science and Technology (Ibolobolo, 2025)



The reactor was operated under ambient tropical conditions characteristic of the study area. Reactor performance was monitored until a steady-state operational regime was achieved prior to granule sampling. Steady-state conditions were defined as a period during which no significant fluctuations in pH and overall reactor performance were observed over successive monitoring cycles. Anaerobic granules were sampled only after operational stabilization to ensure that the microbial community reflected mature and structurally stable granulation.

To enhance anaerobic granulation and microbial activity, the UASB system was supplemented with natural additives, including natural clay, crushed eggshells, ground maize, and lime. These materials were selected based on their potential roles as microbial attachment supports, calcium sources for granule densification, biodegradable carbon sources, and pH-buffering agents, respectively. The effects of these additives on microbial diversity and granulation mechanisms were subsequently evaluated through culture-based microbiological analyses and microscopic observations.

Experimental and Analytical Sites

Experimental Site

The operation and monitoring of the UASB reactor were carried out at the Laboratory of Ecotoxicology, Chemical Safety, and Environmental Biotechnology, Faculty of Science and Technology, University of Kinshasa. Reactor operation, supplementation with natural additives, and routine monitoring were conducted under controlled laboratory supervision to ensure stable operational conditions prior to microbiological sampling.

Microbiological Analysis Site

All microbiological analyses were performed at the Laboratory of Applied Microbiology of Biological and Natural Resources (LaMARBN), Department of Life Sciences, University of Kinshasa. This laboratory is equipped for standard culture-based microbiological techniques, including microbial enumeration on selective and non-selective media, isolation of dominant microbial groups, and light and phase-contrast microscopic observations. These facilities enabled the qualitative and quantitative assessment of cultivable microbial populations associated with anaerobic granules.

Natural Additives Formulation

To stimulate microbial activity and enhance anaerobic granule formation, the UASB reactor was supplemented with selected natural additives based on their physicochemical and biological relevance to anaerobic digestion processes. The selection criteria targeted materials that promote microbial attachment, improve buffering capacity, and stimulate microbial growth and EPS production **(Table 1)**.

Natural Clay

Table 1:
Additives and Their Functional Roles in Anaerobic Granulation

Additive	Primary Functional Role
Natural clay	Provides nucleation sites and increases microbial adhesion surfaces
Crushed eggshells ($CaCO_3$)	Stabilizes pH buffering and provides calcium supplementation
Ground maize	Acts as a readily biodegradable carbon source stimulating microbial growth
Lime	Controls medium acidification and regulates alkalinity

Natural clay was incorporated to enhance microbial attachment and granule nucleation due to its high surface area and surface charge properties. Crushed eggshells served as a source of calcium carbonate, contributing to pH stabilization and granule densification. Ground maize acted as an easily assimilable organic substrate, stimulating microbial proliferation and EPS synthesis. Lime was added to prevent excessive acidification and to maintain favorable anaerobic conditions for microbial metabolic activity.

Sampling and Sample Preparation

Anaerobic granules were aseptically collected from the optimized UASB reactor after steady-state operating conditions had been achieved. Samples were immediately transferred into sterile containers and processed within a tight time frame to minimize alterations in microbial viability and community structure.

Prior to microbiological analysis, granules were gently homogenized and serially diluted in sterile physiological saline solution (0.85% NaCl). Based on preliminary plate count assessments and to avoid colony overgrowth, a 10^{-1} dilution was selected for microbial enumeration, as it consistently yielded plates within the recommended standard countable range of 30–300 colonies per plate (APHA, 2017).

Microbiological Analyses

Microbial diversity and abundance were assessed using culture-based microbiological techniques on selective and non-selective media, targeting the principal functional microbial groups involved in anaerobic digestion and granulation processes **(Table 2)**.

Table 2:
Culture Media and Target Microorganisms

Culture Medium	Target Microorganisms
Chromocult Coliform Agar (CCA)	Total coliforms and *Escherichia coli*
MacConkey Agar	Lactose-positive and lactose-negative *Enterobacteriaceae*
Mannitol Salt Agar (MSA)	Staphylococci (*Staphylococcus aureus* and related species)
Cetrimide Agar	*Pseudomonas aeruginosa*
Sabouraud Dextrose Agar (SDA)	Yeasts and filamentous fungi
Plate Count Agar (PCA)	Total cultivable heterotrophic bacteria

Plates were incubated under targeted temperature and time conditions following standard microbiological protocols (Madigan et al., 2018). Presumptive identification of microbial groups was based on colony morphology, pigmentation, growth characteristics, and selective media responses.

Microscopic Characterization

Microscopic examination of anaerobic granules was conducted to evaluate granule morphology, internal structure, and microbial spatial organization. Granules were gently washed with sterile saline solution and mounted on clean microscope slides. Gram staining and gentian violet staining were applied to differentiate Gram-positive and Gram-negative microorganisms. Observations were performed using phase-contrast optical microscopy at magnifications of 40x and 100x (oil immersion). The following parameters were systematically assessed: granule size and shape, compactness, porosity, microbial aggregation patterns, and the presence of extracellular polymeric substances (EPS).

Calculation of Microbial Density

Microbial abundance was expressed as colony-forming units per milliliter (CFU/mL). Colony counts were performed on plates presenting countable colony ranges, and microbial densities were calculated using standard plate count methods (Mara & Horan, 2003). All microbial enumerations were conducted in triplicate, and mean values were reported.

The microbial density was calculated using the following relationship:

$$\mathrm{CFU/mL} = \frac{N \times D}{V}$$

where:

- N is the number of colonies counted on the plate,
- D is the inverse dilution factor, and
- V is the volume plated (mL).

Statistical Analysis

All experimental data were analyzed using descriptive statistical methods to characterize microbial abundance, relative dominance, and functional composition within anaerobic granules developed in the UASB reactor.

Descriptive Statistics

Microbial enumeration data obtained from triplicate plate counts were expressed as mean values ± standard deviation (SD). Relative abundances (%) of each microbial group were calculated based on their contribution to the total cultivable microbial population.

Data Consistency and Reproducibility

All microbiological measurements were performed in triplicate to ensure analytical precision and reproducibility. Variability among replicate measurements was assessed using standard deviation values.

Statistical Considerations

Given that microbial counts were derived from triplicate measurements of granules collected under a single steady-state operational condition, the statistical analysis was limited to descriptive statistics. No inferential statistical comparisons between independent experimental groups were performed. A conventional significance threshold ($p < .05$) is reported for consistency with microbiological reporting standards, where applicable.

RESULTS

Structural and Microscopic Characterization Results

Granule Morphology and Internal Structure

Microscopic examination revealed that anaerobic granules were predominantly spherical to slightly irregular in shape, with diameters ranging from approximately 1 to 3 mm. The granules exhibited a high degree of compactness and apparent density. Distinct light and dark zones were observed within individual granules, suggesting heterogeneous biomass distribution and the presence of internal voids or diffusion pathways.

Microbial Morphotypes and Community Organization

The granules harbored a structurally heterogeneous microbial community. Filamentous and coccoid bacterial morphotypes were frequently observed and appeared to contribute to cell-to-cell attachment and granule cohesion. Numerous bacterial microcolonies were embedded within a transparent matrix, indicative of substantial extracellular polymeric substance (EPS) production.

Yeasts and filamentous fungi were also detected throughout the granule matrix. Fungal hyphae were occasionally observed forming interconnected networks associated with bacterial aggregates, suggesting a potential role in the degradation of complex organic substrates and in reinforcing the mechanical stability of the granules.

Spatial Distribution of Microorganisms

A stratified spatial organization of microorganisms was evident within the granules. Fermentative bacteria, presumptively identified as *Escherichia coli* and *Klebsiella* spp. based on culture-based results and morphological characteristics, were predominantly localized in the inner layers of the granules. In contrast, other bacterial groups were more frequently observed near the granule periphery, consistent with functional differentiation driven by substrate and metabolite diffusion gradients within anaerobic granules. *Staphylococci* and *Pseudomonas* were distributed primarily along the periphery, promoting surface adhesion and biofilm formation. Fungi (*Aspergillus*, *Penicillium*) were often associated with bacterial filaments, forming supportive structural networks **(Photos 1-3)**.

Photo 1:
Growth of *E. coli* within the granules

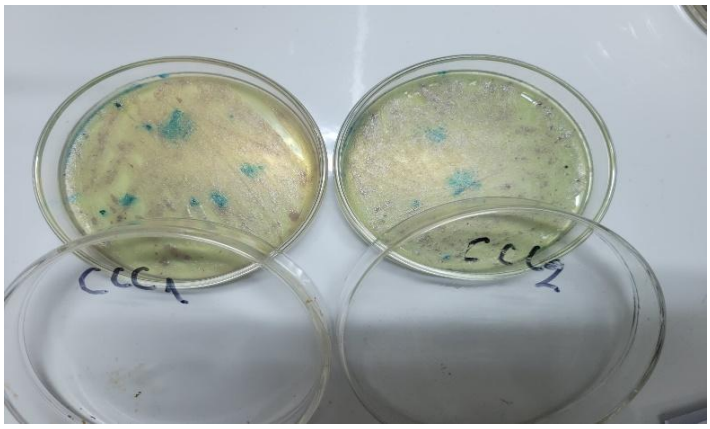


Photo 2:
Growth of Pseudomonas within the granules

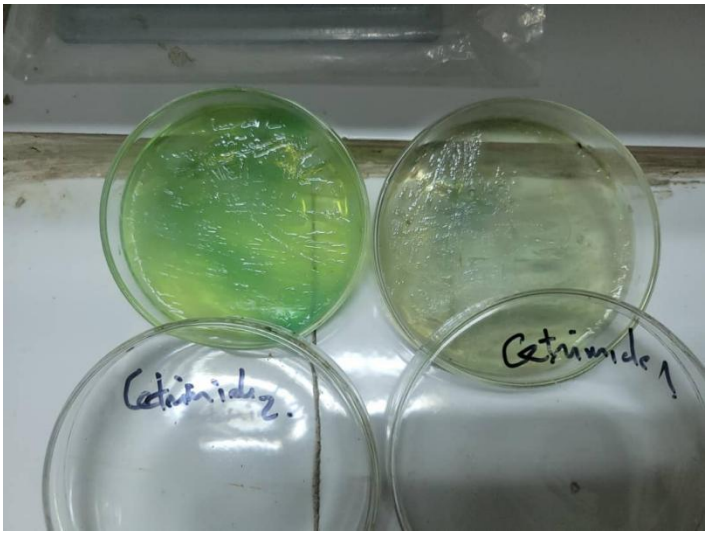


Photo 3:
Growth of Penicillium within the granules

Extracellular Polymeric Substances (EPS) Matrix Evaluation

Phase-contrast microscopic observations consistently revealed the presence of a transparent extracellular matrix surrounding microbial cells and microcolonies within the anaerobic granules. This matrix appeared as refractive or diffracting zones embedded in the granule structure, indicating active EPS localization.

Although EPS concentrations were not quantitatively measured in the present study, their recurrent visual detection suggests active biofilm formation within the granules. The EPS matrix was particularly evident in areas characterized by dense microbial aggregation, supporting its role in cell adhesion, microbial cohesion, and structural stabilization of the anaerobic granules.

Quantitative Microbiological Results

Enumeration of Coliforms and Enterobacteriaceae

The enumeration of coliforms and *Enterobacteriaceae* associated with anaerobic granules is presented in **Table 3**.

Table 3:
Enumeration of Coliforms and Enterobacteriaceae (10^{-1} Dilution)

Medium	Target Microorganism	Colonies Counted (N)	Density (CFU/mL)	Relative Abundance (%)
Chromocult Coliform Agar	*Escherichia coli*	86	8.6×10^3	26.7
MacConkey Agar	Lactose-positive *Enterobacteriaceae* (*E. coli*, *Klebsiella* spp.)	112	1.12×10^4	34.8
MacConkey Agar	Lactose-negative *Enterobacteriaceae* (*Salmonella*, *Shigella*, *Proteus* spp.)	47	4.7×10^3	14.6

Lactose-positive *Enterobacteriaceae* exhibited the highest cultivable density, reaching 1.12×10^4 CFU/mL, followed by *Escherichia coli* with 8.6×10^3 CFU/mL. Lactose-negative *Enterobacteriaceae* were present at lower densities (4.7×10^3 CFU/mL). Overall, fermentative *Enterobacteriaceae* accounted for 76.1% of the total cultivable enterobacterial population, whereas lactose-negative strains represented 14.6%.

Enumeration of Staphylococci

The cultivable staphylococcal population associated with anaerobic granules is summarized in **Table 4**.

Table 4:
Enumeration of Staphylococci (10^{-1} Dilution)

Medium	Target Microorganism	Colonies Counted (N)	Density (CFU/mL)	Relative Abundance (%)
Mannitol Salt Agar	*Staphylococcus aureus*	39	3.9×10^3	12.1
Mannitol Salt Agar	*Staphylococcus epidermidis*	58	5.8×10^3	18.0

Staphylococci were detected at densities ranging from 3.9×10^3 to 5.8×10^3 CFU/mL. *Staphylococcus epidermidis* was more abundant than *S. aureus*, accounting for 18.0% of the total cultivable microbial population, compared with 12.1% for *S. aureus*. Collectively, staphylococci represented approximately 30% of the total cultivable non-fermentative bacterial community associated with the anaerobic granules.

Enumeration of Pseudomonas aeruginosa

The enumeration of biofilm-associated *Pseudomonas aeruginosa* is presented in Table 5.

Table 5:
Enumeration of Pseudomonas aeruginosa (10^{-1} Dilution)

Medium	Target Microorganism	Colonies Counted (N)	Density (CFU/mL)	Relative Abundance (%)
Cetrimide Agar	*Pseudomonas aeruginosa*	26	2.6×10^3	8.1

Pseudomonas aeruginosa was detected at a cultivable density of 2.6×10^3 CFU/mL, representing 8.1% of the total cultivable microbial population associated with the anaerobic granules.

Enumeration of Fungi

The enumeration of yeasts and filamentous fungi associated with anaerobic granules is summarized in Table 6.

Table 6:
Enumeration of Yeasts and Molds (10^{-1} Dilution)

Medium	Target Microorganism	Colonies Counted (N)	Density (CFU/mL)	Relative Abundance (%)
Sabouraud Dextrose Agar	*Candida* spp.	34	3.4×10^3	10.6
Sabouraud Dextrose Agar	Molds (*Aspergillus*, *Penicillium*, *Rhizopus*)	19	1.9×10^3	5.9

Fungi represented a combined relative abundance of 16.5% of the total cultivable microbial population. Yeasts (*Candida* spp.) were more abundant (10.6%) than filamentous molds (5.9%).

Total Cultivable Microbial Population

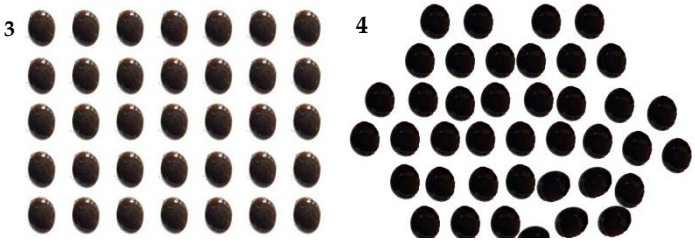
The total cultivable microbial density associated with anaerobic granules on a non-selective medium is presented in Table 7.

Table 7:
Total Microbial Count on Plate Count Agar

Colonies Counted (N)	Total Density (CFU/mL)
165	1.65×10^4

The total cultivable microbial density reached 1.65×10^4 CFU/mL, validating the presence of a robust, viable microbial biomass within the developed granules.

Photo 3 and 4:
Granulation from the optimized UASB reactor



DISCUSSION

Microbial Diversity and Total Density

The total cultivable microbial density in the optimized UASB granules (1.65×10^4 CFU/mL) is consistent with previous studies reporting densities of 10^4–10^5 CFU/mL in stable anaerobic granules treating high-strength wastewater (Hulshoff Pol et al., 2004). This indicates a well-established microbial consortium capable of supporting anaerobic digestion. The dominance of fermentative bacteria (~76%) corroborates the critical role of acidogenesis in initial organic matter breakdown, as highlighted by Lettinga et al. (1997). These microorganisms convert complex sugars and polymers into volatile fatty acids (VFAs), providing essential precursors for downstream methanogenic archaea.

Role of Fermentative Bacteria

The presence of *Escherichia coli*, lactose-positive, and lactose-negative enterobacteria demonstrates high functional diversity among fermenters, enabling the utilization of varied organic fractions present in hospital wastewater. Variations in colony coloration observed on Chromocult Coliform Agar and MacConkey Agar reflect intra-species enzymatic variability, particularly regarding beta-glucuronidase and lactose fermentation profiles (García et al., 2011).

From a functional standpoint, the dominance of these groups ensures robust acidogenesis and VFA production (acetic, propionic, and butyric acids) needed for methanogenesis, directly sustaining long-term granule stability.

Halotolerant and Matrix-Stabilizing Staphylococci

Densities of *Staphylococcus aureus* (3.9×10^3 CFU/mL) and *S. epidermidis* (5.8×10^3 CFU/mL) indicate that staphylococci constitute ~30% of the non-fermentative

microbial community. These bacteria are recognized EPS producers that enhance cell-to-cell adhesion and structural integrity within biological biofilms (Liu et al., 2004). The higher relative abundance of *S. epidermidis* (18.0%) suggests a superior tolerance to fluctuating environmental regimes, such as the local pH and alkalinity shifts induced by the addition of crushed eggshells and lime.

Biofilm-Producing Bacteria: Pseudomonas aeruginosa

Although representing 8.1% of the cultivable population (2.6 x 10^3 CFU/mL), *P. aeruginosa* supports granule structuring by producing highly viscoelastic EPS and forming architectural microchannels. These structural features facilitate substrate diffusion into and gas transport out of the inner granule layers (Flemming & Wingender, 2010). This emphasizes that a microbial group's functional contribution to granulation is not always directly proportional to its numerical abundance.

Fungi: Yeasts and Filamentous Molds

Fungal populations (~16.5%), including *Candida* spp. (10.6%) and various molds (5.9%), confirm active bacterial–fungal coexistence within the granules. Fungi contribute uniquely to the hydrolysis of complex polysaccharides and recalcitrant organic pharmaceutical compounds that bacteria alone cannot efficiently degrade (García et al., 2011; Jiang et al., 2016). Furthermore, structural fungal hyphae weave through the biomass, creating physical networks that anchor bacterial filaments and reinforce mechanical granule stability against fluid shear stresses (Zhao et al., 2018).

Mechanistic Impact of Natural Additives on Granulation

The combined application of natural mineral and organic additives positively influenced microbial density, stratification, and granule stability through discrete mechanisms:

a) **Natural Clay:** Functioned as dense inorganic core nucleation sites, lowering the thermodynamic barrier for initial microbial attachment and increasing the available surface area for biofilm development (Jiang et al., 2016).

b) **Crushed Eggshells and Lime:** Buffered the system within an optimal pH range (7.0–7.5), preventing volatile fatty acid accumulation and maintaining favorable environments for fermentative, halotolerant, and biofilm-forming strains.

c) **Ground Maize:** Served as a slowly releasing, readily biodegradable carbon source that stimulated metabolic pathways linked to microbial proliferation and EPS biosynthesis.

These coordinated inputs supported the development of a functionally diverse, mechanically resilient granule architecture capable of mitigating the toxicity of raw hospital wastewater.

Comparison with Existing Literature

A comparative evaluation of the quantitative microbial densities obtained in this study against historical anaerobic treatment literature is provided in **Table 8**.

Table 8:
Comparative Analysis of Microbial Densities in UASB Granules

Study	Substrate / Operational Conditions	Microbial Density (CFU/mL)	Key Structural Findings
Hulshoff Pol et al. (2004)	Domestic and industrial wastewater	1 x 10^4 to 1 x 10^5	Predominance of fermentative flora
Liu et al. (2004)	UASB with synthetic media + EPS	2 x 10^4	Enhanced biofilm and granule cohesion
Jiang et al. (2016)	UASB + mineral nucleation additives	1.5 x 10^4	Accelerated granule optimization
Present study	Hospital wastewater + natural additives	1.65 x 10^4	Stratified microbial diversity; high stability

The observed microbial densities strongly align with established values, confirming that natural additives can successfully stimulate granule formation and preserve biomass viability even when treating hazardous, complex hospital effluents.

Functional Summary of the Core Consortium

The explicit functional partition established within the optimized UASB granule ecosystem is cross-referenced in **Table 9**.

Table 9:
Functional Stratification Matrix of Microorganisms within the UASB Granules

Microorganism Group	Primary Functional Role in Granulation
Fermentative bacteria (*Enterobacteriaceae*)	Primary hydrolysis, acidogenesis, and VFA precursor production
Halotolerant staphylococci (*S. epidermidis*, *S. aureus*)	Primary EPS matrix secretion, structural cell-to-cell cohesion
Pseudomonas aeruginosa	Structural biofilm reinforcement and nutrient diffusion microchannel formation
Yeasts and molds (*Candida*, *Aspergillus*, *Penicillium*)	Macromolecular polysaccharide cleavage and mechanical hyphal anchoring

https://doi.org/10.4314/orapj.v7i5.49

Limitations and Future Perspectives

While this study offers valuable baseline insights, culture-based enumeration techniques inherently underestimate total microbial diversity by omitting uncultivable anaerobic fractions. Future work should integrate molecular microbial profiling (e.g., 16S rRNA gene and ITS high-throughput sequencing) to map the non-cultivable archaebacterial and methanogenic populations (Wu et al., 2015). Additionally, confocal laser scanning microscopy (CLSM) and metabolomic tracking are recommended to directly visualize bacterial–fungal spatial dynamics and optimize additive dosing sequences under fluctuating hydraulic shock loads.

CONCLUSION

This study provided a comprehensive microbiological characterization of the cultivable microbial diversity within anaerobic granules developed in an optimized UASB reactor treating hospital wastewater at the University Clinics of Kinshasa, utilizing natural additives (clay, eggshells, maize flour, and lime) to enhance granulation.

The integration of diverse microbial populations and engineered natural additives yielded stable, well-structured granules capable of organic matter degradation. The total cultivable biomass reached 1.65 x 10^4 CFU/mL, dominated by fermentative bacteria (~76%), followed by staphylococci and *Pseudomonas* spp. (~30% combined), and functional fungi (~16.5%).

Mechanistically, clay, eggshells, and lime enhanced granule nucleation and pH stabilization, while the ground maize provided an organic carbon stream that stimulated microbial growth and EPS production. The synergistic activity of fermentative bacteria, staphylococci, *Pseudomonas*, and fungi ensured efficient structural cohesion and macromolecular degradation. These findings provide a sustainable, low-cost foundation for wastewater treatment optimization in tropical and low-resource settings, offering an effective strategy for minimizing downstream environmental and public health risks.

Use of Artificial Intelligence (AI) Declaration: No artificial intelligence tools were used in the generation, primary analysis, or interpretation of the experimental raw data presented in this study. All biological experiments, plate counts, and statistical monitoring were executed manually by the authors.

Authors' Contributions: Credo Lialia Mesongolo: Conceptualization, Methodology, Investigation, Data Curation, Formal Analysis, Visualization, Writing – original draft, Project administration. Dieudonné Eyul'anki Musibono: Conceptualization, Supervision, Validation, Resources, Writing – review & editing. Thierry Tabou Tangou: Methodology, Validation, Formal Analysis, Writing – review & editing. Crispin Kyela Mulaji: Investigation, Data Curation, Writing – review & editing. Max Vangu Seke: Formal Analysis, Visualization, Writing – review & editing. Asamboa Shotsha Lionel: Investigation, Methodology, Validation, Writing – review & editing. Emmanuel Makaly Biey: Supervision, Validation, Writing – review & editing.

Data Availability Statement: All data generated or analyzed during this study are included in this published article [and its supplementary information files]. Additional supporting data are available from the corresponding author upon reasonable request, subject to institutional policies.

Acknowledgements: The authors express their sincere gratitude to the Cliniques Universitaires de Kinshasa for authorizing hospital wastewater sampling. We acknowledge the technical support and supervision provided by the staff of the Laboratory of Ecotoxicology, Chemical Safety, and Environmental Biotechnology during UASB reactor operation and monitoring. Special thanks are extended to the members of the Laboratory of Applied Microbiology of Biological and Natural Resources (LaMARBN) for their invaluable assistance in microbiological analyses, colony enumeration, and scientific expertise.

Ethical Approval: Ethical approval was not required for this study as it did not involve human participants, animals, or endangered species. All fieldwork and sampling activities were conducted in strict accordance with national environmental regulations and institutional guidelines.

Informed Consent: Nil required.

Conflicts of Interest: The authors declare that they have no conflicts of interest.

Funding: This research received no specific grant from any funding agency in the public, commercial, or not-for-profit sectors.

ORCID iDs:

Mesongolo, C. L.[1]: https://orcid.org/0009-0002-1970-7762
Musibono, D. E.[1]: https://orcid.org/0009-0003-9739-8333
Tangou, T. T.[1]: https://orcid.org/0009-0005-2766-3060
Mulaji, C. K.[2]: https://orcid.org/0000-0002-9406-3277
Seke, M. V.[3]: https://orcid.org/0009-0007-4886-1132
Asamboa, L. S.[4]: https://orcid.org/0009-0005-2633-5348
Biey, E. M.[1]: https://orcid.org/0009-0005-0790-6627

REFERENCES

APHA. (2017). *Standard methods for the examination of water and wastewater* (23th ed.). American Public Health Association, American Water Works Association, Water Environment Federation.

Flemming, H.-C., & Wingender, J. (2010). The biofilm matrix. *Nature Reviews Microbiology*, *8*(9), 623–633. https://doi.org/10.1038/nrmicro2415

García, J., Ferrer, J., & García-Heras, J. L. (2011). Microbial diversity and activity in anaerobic granular sludge: Implications for wastewater treatment. *Bioresource Technology*, *102*(12), 6813–6820. https://doi.org/10.1016/j.biortech.2011.03.072

Hulshoff Pol, L. W., van der Lans, R. G. J. M., & Lettinga, G. (2004). Anaerobic granular sludge: Microbial structure and activity in UASB reactors treating industrial and domestic wastewaters. *Water Science and Technology*, *49*(11–12), 1–9. https://doi.org/10.2166/wst.2004.0337

Jiang, M., Lu, H., Yang, F., & Li, Y. (2016). Enhancement of anaerobic granulation using natural mineral additives in UASB reactors. *Bioresource Technology*, *220*, 55–63. https://doi.org/10.1016/j.biortech.2016.08.008

Lettinga, G., van Velsen, A. F. M., Hobma, S. W., de Zeeuw, W., & Klapwijk, A. (1997). Use of the upflow anaerobic sludge blanket (UASB) reactor concept for biological wastewater treatment. *Biotechnology and Bioengineering*, *22*(4), 699–734. https://doi.org/10.1002/bit.260220412

Liu, Y., Whitman, W. B., & Boone, D. R. (2004). Role of EPS and co-aggregating bacteria in granule formation. *Applied and Environmental Microbiology*, *70*(3), 1333–1340. https://doi.org/10.1128/AEM.70.3.1333-1340.2004

Madigan, M. T., Martinko, J. M., & Bender, K. (2018). *Brock biology of microorganisms* (15th ed.). Pearson.

Mara, D., & Horan, N. (2003). *The handbook of water and wastewater microbiology* (2nd ed.). Academic Press.

Verlicchi, P., Galletti, A., Petrović, M., & Barceló, D. (2010). Hospital effluents as a source of emerging contaminants: An overview of micropollutants and sustainable treatment options. *Journal of Hydrology*, *389*(1–2), 416–428. https://doi.org/10.1016/j.jhydrol.2010.06.005

Wu, W.-M., Wang, A., & Huang, J. (2015). Microbial interactions in anaerobic granules: Implications for stability and performance of UASB reactors. *Water Research*, *68*, 452–462. https://doi.org/10.1016/j.watres.2014.10.038

Zhao, J., Zhang, Q., & Li, X. (2018). Fungal contribution to anaerobic granulation and organics degradation in UASB reactors. *Bioresource Technology*, *249*, 234–243. https://doi.org/10.1016/j.biortech.2017.10.107